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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EV 326917243 US

INVENTOR(S)

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Steven	Tinge	Lebanon, Illinois

Additional inventors are being named on the 1 *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Live, Attenuated Bacterial Vectored Vaccines against Anthrax and Plague

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification *Number of Pages* 6 ☐ CD(s), Number ______

☑ Drawing(s) *Number of Sheets* 3 ☐ Other (specify) ______

☐ Application Date Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☐ Applicant claims small entity status. See 37 CFR 1.27.

☑ A check or money order is enclosed to cover the filing fees. (check no. 5104)

☐ The Director is herby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 50-0268

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FILING FEE Amount ($): 160.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.

☑ Yes, the name of the U.S. Government agency and the Government contract number are: Department of Defense (JVAP), subcontract under DynPort Vaccine Co., LLC, no.DPSC-02-02257

[Page 1 of 2]

Respectfully submitted,

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Date April 2, 2004

REGISTRATION NO. 30,237
(if appropriate)

Docket Number: AVA-434.1 PRV

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

PROVISIONAL APPLICATION COVER SHEET
Additional Page

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Docket Number AVA-434.1 PRV

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Number 1 of 1

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Tinge, et.al.	
Serial No.:	(not yet assigned)	Art Unit:
Filed:	concurrently herewith	Examiner:
Entitled:	LIVE, ATTENUATED BACTERIAL VECTORED VACCINES AGAINST ANTHRAX AND PLAGUE	

Attorney Docket No.: AVA-434.1 PRV

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1. Provisional Application for Patent coversheet (PTO/SB/16) 2 page(s), in duplicate
2. Specification of 6 pages
3. Drawings (Informal) of 3 Sheets
4. Check No. 5104 in the amount of $ 160.00 to cover provisional patent application filing fee.
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Express Mailing Label No. = EV 326917243 US

Date of deposit: April 2, 2004

by Nasim G. Memon

Live, Attenuated Bacterial Vectored Vaccines against Anthrax and Plague

Inventors: Steven Tinge, Sims Kochi, Ken Roland, Donata Sizemore, Hedy Adari, Lawrence Thomas, Kevin P. Killeen

Introduction

An outbreak in 1979 of human anthrax in the former Soviet city of Sverdlosk was shown to be the result of an accidental release of weaponized spores from a military production facility. The mailing of anthrax spores in the U.S. in 2001 further demonstrated that an aerosolized, biological weapon could be effectively deployed against a civilian population. Inhalation anthrax and pneumonic plague are caused by infections initiating on mucosal surfaces of the respiratory tract and are the deadly forms of their respective diseases. The high mortality rate in individuals that contract inhalational forms of infections like anthrax and plague, and the apparent ease with which these select agents may be weaponized ("Category A" biological weapons) have hastened renewed efforts to develop vaccines to protect against microbes adaptable as agents of biowarfare and terrorism.

The development of vaccines to protect against biological weapons has largely focused on parenteral administration that elicits the production of systemic (i.e., immunoglobulin G, or IgG) and not mucosal (secretory IgA) antibodies. Despite advances in understanding the mechanisms by which organisms such as *Bacillus anthracis* (anthrax) and *Yersinia pestis* (plague) cause disease, sustained efforts to develop new vaccines to protect against infection have been relatively modest. The only anthrax vaccine approved for use in the United States (Anthrax Vaccine Adsorbed [AVA]; BIOTHRAX™), for example, requires six injections over

18 months with yearly boosters. Pre-clinical immunogenicity and efficacy studies evaluating BIOTHRAX™ suggested that the presence of IgG to the anthrax toxin protective antigen (PA) correlated with protection. Additional studies demonstrated that antibody capable of neutralizing PA activity *in vitro* was also a reliable surrogate marker for protection. The manufacture of a vaccine against plague was recently terminated following concerns over reactogenicity of the vaccine and its questionable efficacy against the pneumonic origin of the disease. Pre-clinical immunogenicity and efficacy studies evaluating candidate plague vaccines (based on *Y. pestis* F1 capsule, V antigen, or a F1-V fusion protein) demonstrated that serum IgG is a reliable correlate of protection against experimental plague challenge.

Summary of the Invention

The present invention relates to the development of orally administered anthrax and plague vaccine candidates utilizing attenuated *Vibrio cholerae* and *Salmonella spp.* vector technologies. The development of these vectors capitalizes on their established safety and immunogenicity profiles in humans as orally administered, live, attenuated vaccines against enteric infections such as cholera (CholeraGarde®, *V. cholerae* Peru-15), typhoid fever (*S. typhi* Ty800), and non-typhoidal salmonellosis (*S. typhimurium* LH430). It is an object of the present invention to provide oral, preferably single-dose, vector vaccines that rapidly confer protective immunity against biological weapons agents and that are superior to injectable vaccines.

Several approaches have been pursued in an effort to optimize the presentation of anthrax and plague antigens to the immune system by recombinant Peru-15, Ty800, and LH430. These vectors have been engineered to express PA, F1, V, or a F1-V fusion (antigens previously shown to provide different levels of protection against infection) to various cellular locations, e.g., cytosol, surface, secreted. To provide stabilized, high-level antigen expression, we developed host-vector systems employing balanced-lethal plasmids: a vector bearing a lethal mutation is rescued by a multi-copy, bi-functional plasmid that complements the mutation and encodes the vaccine antigen. Use of this system in attenuated Salmonella and Vibrio precludes the need for antibiotic selection and has provided increased stability of the expression plasmid and the encoded heterologous antigen. The development of multiple, balanced-lethal, host strains also

provides the ability to evaluate recombinant antigen-expressing plasmids simultaneously in attenuated *S. typhimurium* and *V. cholerae* strains.

Bacterial-vectored anthrax vaccine candidates

We constructed a Δ*asd* (aspartate semi-aldehyde dehydrogenase) mutant of *S. typhimurium* LH430 (M015) to secrete PA into the extracellular environment as a fusion to the N-terminus of *E. coli* hemolysin ($HlyA_s$). Strain M015 expressing the $HlyA_s$-PA_{63} fusion from a bi-functional Asd^+ plasmid (pMEG-1672) was genetically stable and secreted high levels of the fusion protein into the culture medium (Figure 1A). We also produced several Δ*glnA* (glutamine synthetase) vectors based on attenuated *V. cholerae* serotypes. One of these strains, Peru-15Δ*glnA*, bearing a bifunctional $GlnA^+$ plasmid encoding an Hly-PA fusion (pMEG-1787) secreted Hly-PA_{63} into the culture medium (Figure 1B).

Three- to four-week old, germ free, Swiss-Webster mice were orally immunized with Peru-15Δ*glnA*/pMEG-1787 on days one, three, and eight. Fourteen days later (study day 22), animals were boosted with a subcutaneous injection of recombinant PA (2.5 μg per dose) formulated with Alhydrogel. Serum samples recovered on day 28 revealed that five of 12 immunized mice developed anti-PA titers greater than those observed from mice receiving the vaccine control (no PA_{63}) followed by a rPA booster injection.

To enhance heterologous antigen expression, we vectored a codon-optimized PA gene into attenuated strains of *Vibrio* and *Salmonella*. The Western blot in Figure 2 shows a significant increase in PA expression by both vectors.

Bacterial-vectored plague vaccine candidates.

Twenty attenuated *S. typhimurium* and six attenuated *V.cholerae* vaccine vector strains were constructed to express either the F1 antigen, V antigen or F1-V fusion antigens derived from *Y. pestis*. *In vitro* studies identified an attenuated strain of *S. typhimurium*, M020, expressing a genetically stable fusion of the F1 and V antigens (F1-V) in the cytoplasm from an Asd^+ balanced-lethal plasmid (Figure 3).

Two doses of M020 were administered to mice by oral feeding on day 0 and 14 and the immune response was measured two and four weeks following the second immunization. Reciprocal antibody titers specific to F1-V ranged from 800-12,800 and 600-19,200 at two and four weeks post-boost, respectively (**Table 1**). These results indicate that M020 expressed F1-V *in vivo* that elicited the production of high levels of F1-V specific antibody. The same experiment compared various balanced-lethal vector systems and their effect on the capacity of a *S. typhimurium*-based vaccine to elicit the production of antibodies to F1-V.

Table 1. Immunogenicity of *Salmonella typhimurium* M020 in BALB/c Mice

Mouse #	2wks post-boost Serum IgG Anti- F1	4wks post-boost Serum IgG Anti- F1	2wks post-boost Serum IgG Anti- V	4wks post-boost Serum IgG Anti- V	2wks post-boost Serum IgG Anti- F1-V	4wks post-boost Serum IgG Anti- F1-V	2wks post-boost Serum IgG LPS
21	100	150	400	800	800	800	0.049
22	100	150	400	600	1000	800	0.353
23	200	9600	800	1600	3200	6400	0.405
24	<100	100	200	200	800	600	0.040
25	200	600	1600	4800	4800	4800	0.454
26	<100	100	2400	4800	12800	6400	1.219
27	300*	800	600	400	3200	800	0.340
28	100	4800	4800	12800	12800	19200	0.688
29	200	800	400	400	1200	1200	0.492
30	100	150	200	400	800	800	1.055
GMT	**137**	**476**	**685**	**1008**	**2341**	**2017**	**AVG= 0.510**

*Reciprocal endpoint dilution

A comparison of the M020 balanced-lethal system (Asd^{+}) to systems we had developed in *S. typhimurium* based on GlnA^{+} and PurB^{+} revealed that the former induced higher serum antibody responses to F1-V than the latter two (**Table 2**).

Table 2. Immunogenicity of *S. typhimurium* Vector Vaccines Expressing Plague F1-V Antigen (GMT of Reciprocal Titers)

Inoculum (1 x 10^{9} cfu/mouse)	Host deletion	2 wks post-boost Serum IgG Anti- F1	4 wks post-boost Serum IgG Anti- F1	6 wks post-boost Serum IgG Anti- F1	9 wks post-boost Serum IgG Anti- F1	2 wks post-boost Serum IgG Anti- V	4 wks post-boost Serum IgG Anti- V	6 wks post-boost Serum IgG Anti- V	9 wks post-boost Serum IgG Anti- V	2 wks post-boost Serum IgG Anti- F1-V	4 wks post-boost Serum IgG Anti- F1-V	6 wks post-boost Serum IgG Anti- F1-V	9 wks post-boost Serum IgG Anti- F1-V
Salmonella typhimurium	*purB/*	100	149	149	123	217	226	141	141	363	386	246	264
Salmonella typhimurium	*glnA*	104	214	141	152	256	393	115	115	650	627	230	264
Salmonella typhimurium (M020)	*asd*	137	476	132	132	685	1008	123	162	2341	2017	230	303

The possibility of inducing an enhanced immune response was evaluated by varying the time between primary and booster immunizations and employing other *Salmonella* vaccine constructs that expressed only F1 or V antigen. Several of these optimized dosing regimens elicited enhanced anti-F1 and anti-V immune responses. cGMP Reseach-, Master- and Working-Seed Banks of plague vaccine candidate M020 were produced.

The severity of disease and infrequent occurrence of pneumonic plague in the U.S. renders plague vaccine challenge and efficacy studies untenable in humans. To address this issue, the FDA recently modified the Code of Federal Regulations (CFR) to include the "Animal Rule" (21 CFR 601.90-95), which enables the licensure of counter-bioterrorism vaccines, like those against plague, following appropriate safety and immunogenicity studies in humans and efficacy studies in two animal species. Accordingly, steps are being taken to explore the clinical pathway of M020 in humans and efficacy pathway in animals.

Abstract of the Disclosure

Novel vectors for immunizing against *Y. pestis* and *B. anthracis* antigens are disclosed which are useful as vaccines against plague and anthrax. Strains of *Vibrio cholerae*, *Salmonella typhi*, and *Salmonella typhimurium* having attenuating mutations are disclosed for use as vectors of plague and anthrax antigens, e.g., *Y. pestis* F1 and V antigens, F1-V fusion protein, and *B. anthracis* Protective Antigen. A *S. typhimurium* mutant strain, designated M020, showing expression of high levels of F1-V *in vivo* and eliciting high titre anti-F1-V IgG responses is particularly disclosed. Such vectors provide candidates for oral vaccines against airborne plague and anthrax pathogens.

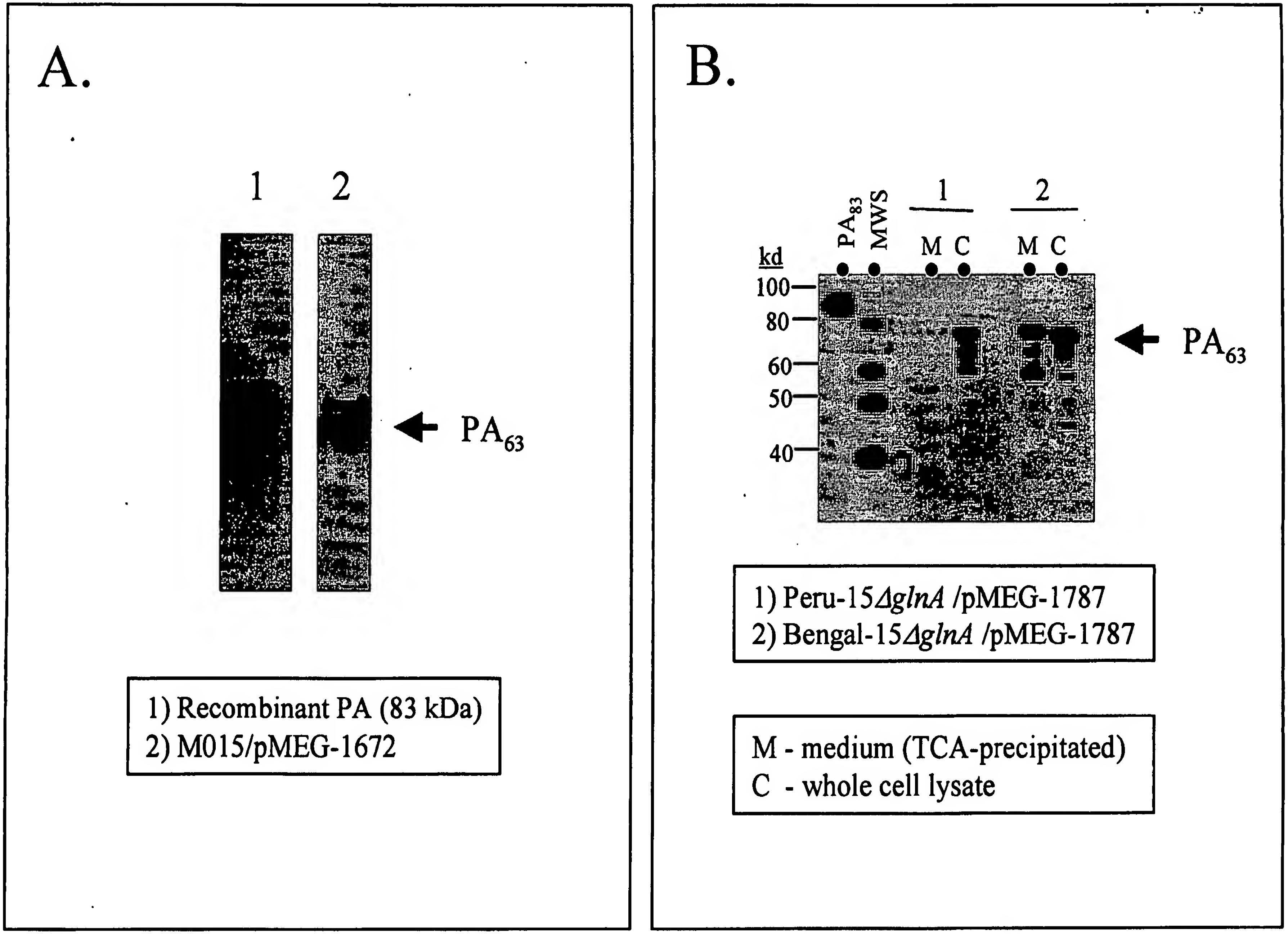
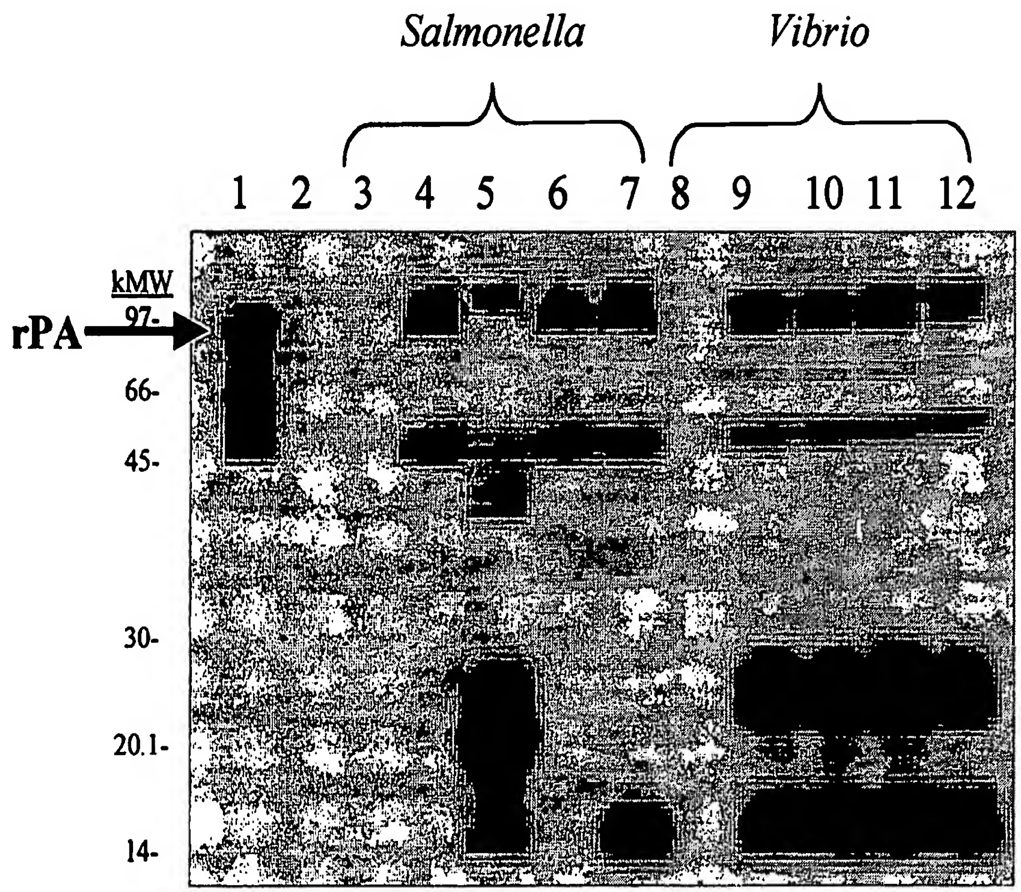
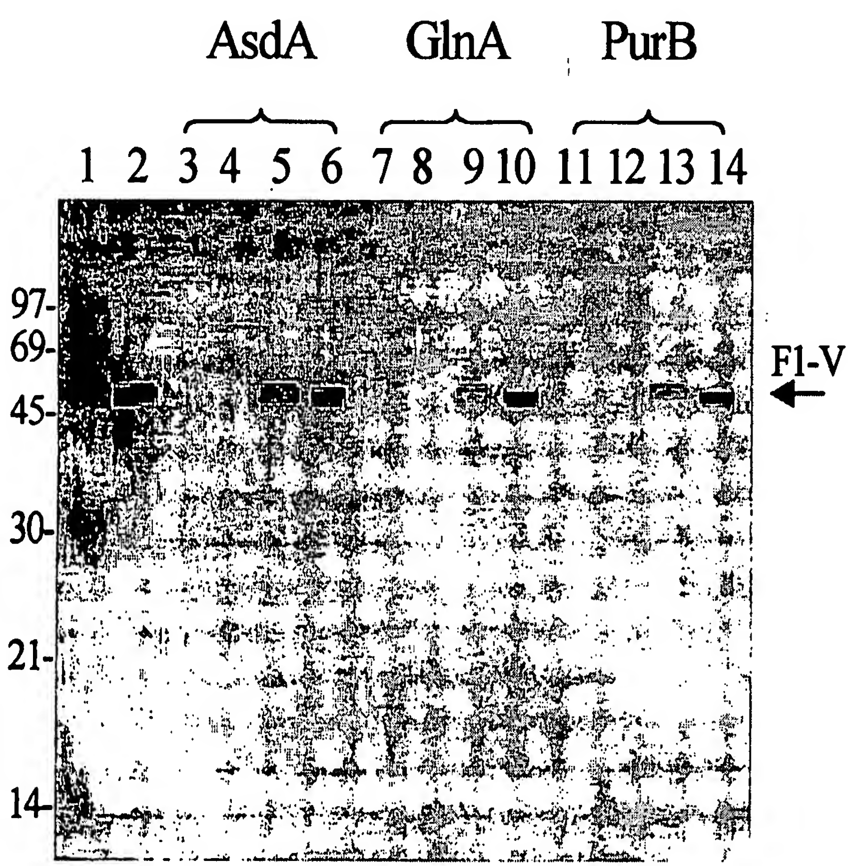


Figure 1. Western Blot Analysis of the Expression of HlyAs-PA$_{63}$ by Attenuated *S. typhimurium* (A) and *V. cholerae* (B)



Lane	Sample
1	rPA (.1 ug)
2	HMW Marker
3	*S. typhimurium* negative control
4-7	*S. typhimurium* PelB-PA (GlnA^{+})
8	*V. cholerae* negative control
9-12	*V. cholerae* PelB-PA (GlnA^{+})

Figure 2. Whole Cell Expression of PelB::PA Optimized from pBR Based Gln+ Vector in *S. typhimurium* LH430 and *V. cholerae* Peru-15 Derivatives



Lane	Sample			
1	High Molecular Weight Marker			
2	rF1-V, 0.5ug			
3	MGN6476	S	(vector only)	AsdA
4		I		AsdA
5	**M020**	S	(+F1-V)	AsdA
6		I		AsdA
7	MGN7267	S	(vector only)	GlnA
8		I		GlnA
9	MGN7258	S	(+F1-V)	GlnA
10		I		GlnA
11	MGN7327	S	(vector only)	PurB
12		I		PurB
13	MGN7325	S	(+F1-V)	PurB
14		I		PurB

S= soluble fraction I= insoluble fraction

Figure 3. Western Blot Analysis of F1-V Expression by Attenuated *S. typhimurium* Asd$^+$, Gln$^+$, and Pur$^+$ Balanced-lethal Vectors.

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